OVERPRODUCTION OF HYDROGEN FROM AN ANAEROBIC BACTERIUM

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ABSTRACT

A derivative of the anaerobic bacterium *Clostridium phytofermentans* was isolated and shown to produce two moles of hydrogen per mole of glucose and similar yields from cellulosic feedstocks. The strain (cpnit-1) was selected for its rapid growth in a chemostat under nitrogen fixation conditions. The rare ability of cpnit-1 to stably, anaerobically produce hydrogen while rapidly fixing its own nitrogen provides a strong selection to maintain the culture and suggests a uniquely simple hydrogen reactor design based on renewable feedstocks.

1. INTRODUCTION

Hydrogen is an ideal fuel since its only oxidation product is water. When used in a fuel cell to generate electricity, it is three times as efficient as an internal combustion engine. However, its production, primarily from steam reformation of natural gas at 700-1100^{o} C ($CH_4 + H_2O \rightarrow CO + 3H_2$), requires much more energy than is created. Hydrogen can also be produced by electrolysis, splitting water into its component gases, hydrogen and oxygen. However, the electrical demand for that process also far exceeds the energy value of the resulting hydrogen. Biological hydrogen production, typically using photosynthetic algae or anaerobic bacteria, is an ambient temperature, catalytic process with the potential for a significant net energy gain. In order to be scalable, the process must be stable, and in order to be economical and sustainable it must provide a high yield of hydrogen from renewable feedstocks.

Clostridium phytofermentans is a recently-discovered anaerobic bacterium, reported to possess cellulase enzymes that degrade cellulose polymers to fermentable substrates, and other enzymes to ferment starch and both five and six carbon sugars derived from hemicellulose and cellulose, respectively (Warnick et al., 2002). These biochemical capabilities enable *C. phytofermentans* to degrade all the essential components of plant material and make it a candidate for consolidated bioprocessing, an as-yet-undemonstrated process by which plant material might be converted to fuel, essentially in a single step.

Biological nitrogen fixation provides about 40% of the nitrogen found in the world's soil and water (Postgate, 1998). Relatively few (perhaps 100) bacteria possess this capability, in which atmospheric nitrogen (N_2) is reduced to ammonia via the nitrogenase enzyme. The reaction is of great economic importance since fully 1% of the world's energy supplies are consumed in the industrial fixation of nitrogen (Haber-Bosch process), mostly to produce fertilizer. Nitrogenase provides a catalytic alternative to the commercial fixation of nitrogen at a time when fertilizer has increased tremendously in price, primarily due to the cost of the natural gas from which it is made. The stability of a biological hydrogen reactor could be enhanced significantly by using a rapidly growing organism capable of fixing its own nitrogen, since few microbial contaminants can successfully compete.

Nitrogenase also produces hydrogen in addition to ammonia (one mole of H_2 per mole of N_2 fixed). A nitrogen-fixing, hydrogen-producing strain offers the potential to link these two processes, thereby positively impacting the economics of both.

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2. MATERIALS AND METHODS

Details on growth of the bacteria: Lyophilized *C. phytofermentans* was initially inoculated into chopped beef medium. When vigorous growth was established, the organism was switched to a mineral salts medium consisting of 1 g/L glucose or cellobiose, 29 g/L K_2HPO_4, 15 g/L KH_2PO_4, 0.5 g/L Na_2SO_3, 0.002 g/L adenine, 0.005/L g cytosine, 0.002 g/L guanosine, 0.04/L g uracil, 0.005/L g thymine, 0.02 mg/L biotin, 0.02 mg/L folic acid, 0.1 mg/L pyridoxine-HCl, 0.05 mg/L thiamine-HCl, 0.05 mg/L riboflavin, 0.05 mg/L nicotinic acid, 0.05 mg/L calcium panthothenate, 0.0001 mg/L vitamin B12, 0.05 mg/L p-aminobenzoic acid, 0.01 mg/L thioctic acid. A mineral salts solution was made separately and added to the medium with the following final concentrations: 5 mg/L citric acid, 62 mg/L $MgSO_4 \cdot 7H_2O$, 5.5 mg/L $MnSO_4 \cdot 4H_2O$, 10 mg/L NaCl, 1 mg/L $FeSO_4 \cdot 7H_2O$, 1.7 mg/L $CoCl_2 \cdot 6H_2O$, 1.3 mg/L $CaCl_2 \cdot 2H_2O$, 1.8 mg/L $ZnSO_4 \cdot 7H_2O$, 0.5 mg/L $CuSO_4 \cdot 2H_2O$, 0.5 mg/L $AlK(SO_4)_2 \cdot 12H_2O$, 0.1 mg/L H_3BO_4, 0.1 mg/L VCl2, and 0.11 mg/L $NaMoO_4 \cdot 2H_2O$. Optimal growth was obtained at about 34° C and pH 7. Bioreactor studies were conducted in New Brunswick BioFlo 110 5 L vessels. Nitrogen, argon or helium was sparged through the bioreactor to maintain anaerobicity and, in the case of nitrogen, to serve as the sole source of nitrogen for growth. Hydrogen detection was accomplished with a HY-OPTIMA™ 700 in-line process hydrogen monitor, sealed in a separate vessel connected via glass tubing to the reactor headspace. The monitor was factory calibrated with appropriate gas standards.

3. RESULTS

Previous studies by Warnick et al. with *C. phytofermentans* reported its growth on complex medium including tryptone as a nitrogen source[1]. We began studies in our laboratory by developing a chemically defined medium on which to grow the organism; i.e. a medium containing only the compounds essential to support growth (C,H,O,N,P,S sources, and various trace inorganics and nutrients, detailed in MATERIALS AND METHODS, above). We started with a single colony isolate of *C. phytofermentans*. In the course of determining its nitrogen utilization capabilities, we made the fortuitous discovery that it was capable of fixing its own nitrogen. Specifically, we observed vigorous growth when nitrogen gas was supplied as the sole source of nitrogen for growth, and no growth in a parallel culture sparged with either argon or with helium.

Although we were unable to detect hydrogen from the native strain, we hypothesized that a strain with enhanced nitrogenase activity might produce detectable hydrogen. Towards this end, the native strain was inoculated into a 5 L chemostat continuous culture with N_2 gas as the sole source of nitrogen for growth. These conditions were designed to select for increased nitrogenase activity since N_2 utilization was the presumptive limitation on growth. The culture was continuously UV-irradiated at 254 nm in an enclosed loop, simultaneously selecting for high growth rate and for phenotypic stability, both of which are critical characteristics for a biological hydrogen reactor.

After four months of continuous irradiation and selection, a fast-growing variant was isolated. The strain, cpnit-1, showed a 33% growth rate advantage over the native strain (Figure 1).

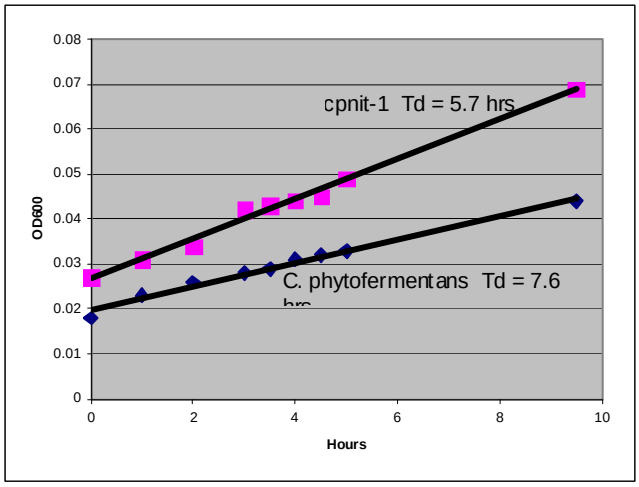

Figure 1. Comparative growth rates of cpnit-1 and its *C. phytofermentans* parental strain.

In consideration of the possibility that cpnit-1 might represent a contaminant rather than a derivative of *C. phytofermentans*, gas chromatography fatty acid (GCFA) analysis was conducted on both strains. Although *C. phytofermentans* is not yet included in the GCFA database, Figure 2 shows the essential coincidence of the major chromatographic peaks from cpnit-1 and the parent strain, providing strong evidence that they represent organisms of similar derivation.

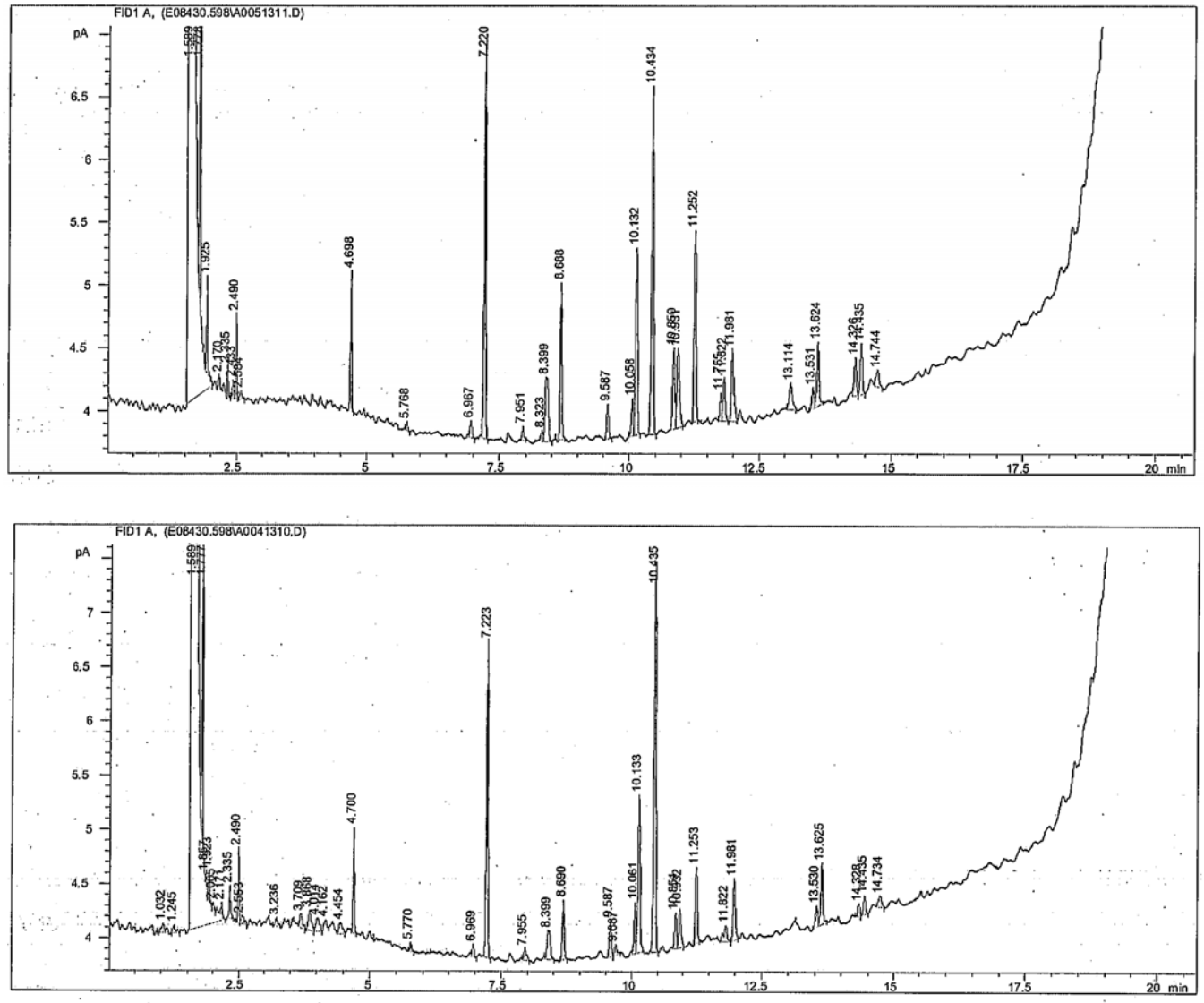

Figure 2. Gas chromatography fatty acid analysis of parent strain (top) and cpnit-1 (bottom).

Table 1 shows the hydrogen yields when cpnit-1 was grown on various pure carbon sources. Hydrogen yield was typically between two and three moles of hydrogen per mole of glucose equivalent. This value represents 50-75% of the maximum theoretical yield of four moles per mole of glucose (which is unattainable in actual practice). When argon was substituted for nitrogen as the carrier gas (in a culture previously exposed to nitrogen), the hydrogen yield was less than 1% that observed with nitrogen, although the cells still grew to an optical density (600 nm) of about 0.16, which is about 30% of the density typically attained with a nitrogen gas purge. The cells had apparently stored enough organic nitrogen for this level of growth. This result strongly implicates the nitrogenase enzyme in the production of hydrogen, since nitrogenase would have been active during growth on nitrogen and inactive during growth on argon.

Table 1. Hydrogen yield.

Carbon source	Carrier Gas	Flow Rate (ml/min)	Moles H_2/ Glucose Equiv.
Glucose	Nitrogen	20	3.02
Glucose	Nitrogen	20	1.99
Cellobiose	Nitrogen	20	4.08
Cellobiose	Nitrogen	20	2.83
Glucose	Argon	1	0.005

A typical hydrogen/growth profile is shown in Figure 3.

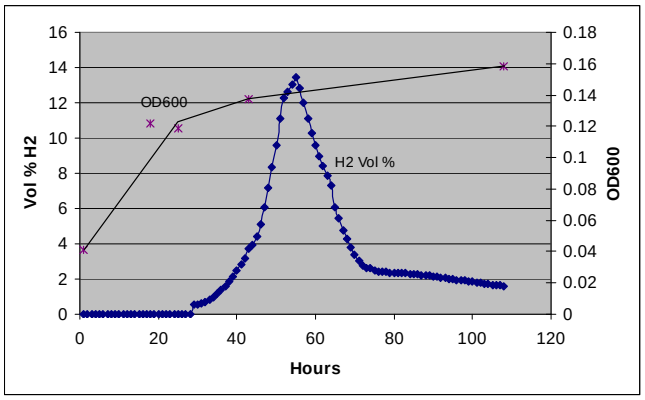

Figure 3. Hydrogen production from cpnit-1.

4. DISCUSSION AND CONCLUSIONS

A naturally-selected, fast-growing derivative of *C. phytofermentans* was isolated that produces significant amounts of hydrogen from renewable materials while fixing its own nitrogen. To our knowledge this approach to the enhancement of biological hydrogen production is a novel one.

The nitrogenase enzyme, in addition to fixing nitrogen, nitrogenase also produces hydrogen at the rate of one mole per mole of N_2 fixed. The overall reaction catalyzed by nitrogenase can be expressed as (Eq. 1)

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16\ Pi \quad (1)$$

The production of hydrogen in the presence of nitrogen by cpnit-1, and the relative lack of hydrogen production (<1%) when nitrogen was replaced with argon, implicate the nitrogenase enzyme in the production of hydrogen in this system. This is consistent with the starting hypothesis of this work; that enhancement of nitrogen fixation would lead to increased hydrogen production. Proteomics and molecular genetics approaches are currently underway to define this further.

Because the selection used for cpnit-1 simultaneously selects for phenotypic stability, the typical genetic instability associated with most molecular genetics constructs is avoided. Repeated, continuous fermentations with cpnit-1 over many weeks, with occasional single colony isolations have revealed no evidence of contamination or decrease in hydrogen production. Presumably this is because the rapid growth rate and unique nutritional requirements, especially the fixation of nitrogen, provide a continual positive selection during fermentation. These results suggest it may be possible to maintain cpnit-1 in culture indefinitely without the need to ensure a sterile feed. This would greatly simplify and reduce the costs biological hydrogen production.

It has also proven feasible to produce hydrogen from processed *Phragmites australis* (common reed), which grows essentially as a weed around waterways around the globe. We were particularly interested in utilization of *Phragmites australis* because it is an invasive species that grows globally around waterways, including on many U.S. military installations. The plant takes up nitrogen and phosphorus from the water, so its harvesting effectively removes the nitrogen and phosphorus from the water where it would otherwise lead to eutrophication, and makes the material available for processing into hydrogen.

The unique qualities of cpnit-1 suggest an exceptionally simple and potentially transportable design for a biological hydrogen reactor (Figure 4). A tank, sized to need, receives a slow sparge of nitrogen, a cellulosic feedstock, water with trace inorganic salts (possibly wastewater), and an inoculation of cpnit-1. The nitrogen sparge maintains anaerobicity, supports growth and purges the headspace of hydrogen, which is separated from the other gases by a palladium membrane or other hydrogen-selective membrane, then compressed and stored for use. Since cpnit-1 grows rapidly and uses N_2 as its sole source of nitrogen, it out-competes other organisms to maintain the stability of the reactor. This simple, low-energy reactor design coupled with renewable feedstocks and the selection provided by cpnit-1's nitrogen and carbon utilization should provide a stable means to continuously produce hydrogen at ambient temperatures from renewable materials, while simultaneously producing organic nitrogen suitable for fertilizer use.

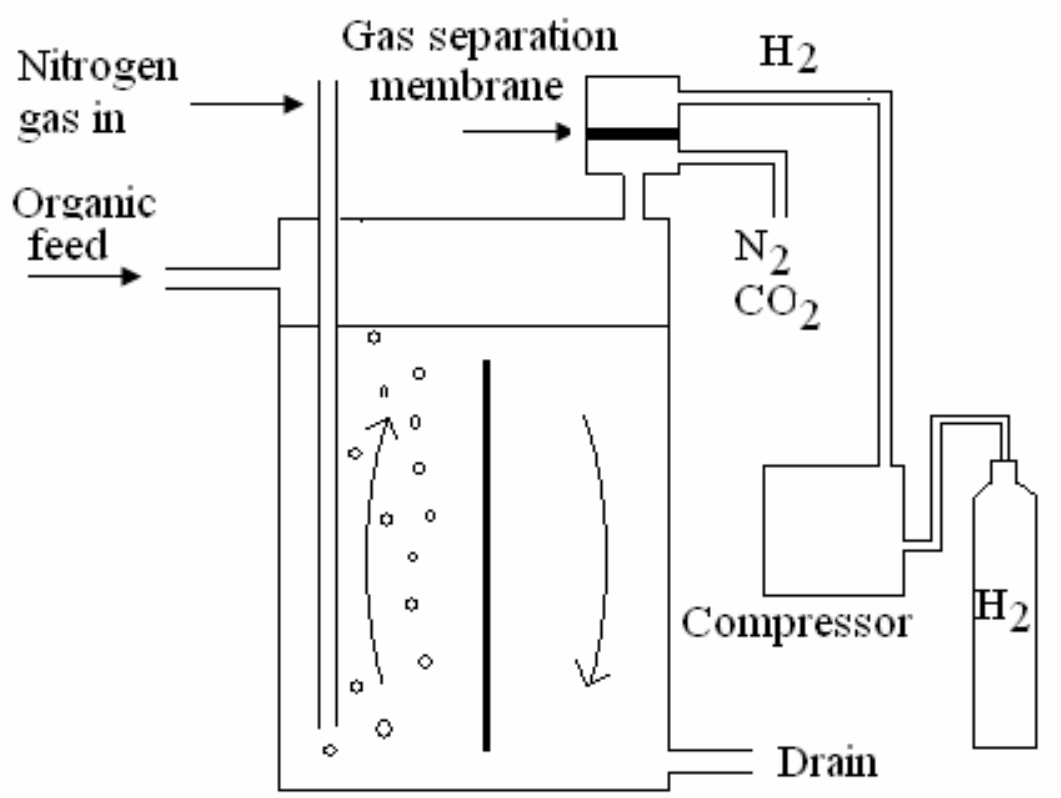

Figure 4. cpnit-1 biological hydrogen reactor.

In summary, the economics of this unique process are positively impacted by the high level of hydrogen production, the use of the invasive, renewable and perennial species *Phragmites australis* as feedstock, and the use of inexpensive nitrogen gas as the sole source of nitrogen for growth. The process itself is strongly enhanced by the genetic stability of cpnit-1 and the apparent lack of need to maintain sterility of the feed. And the environment is protected by the removal of nitrogen and phosphorus from waterways, the production of clean hydrogen fuel, and the simultaneous biological fixation of nitrogen, subsequently suitable for use as fertilizer.

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